

Appln No.: 10/828,395

Amendment Dated: May 11, 2007

Reply to Office Action of February 28, 2007

#### REMARKS/ARGUMENTS

This is in response to the Office Action mailed February 28, 2007 for the above-captioned application. Reconsideration and further examination are respectfully requested.

The typographical error in claim 11 noted by the Examiner has been corrected.

Claims 1, 6 and 11 stand rejected under 35 USC § 112, first paragraph, as lacking written description. The Examiner states that there is no written description for agents other than oligonucleotides. Applicants respectfully disagree. ¶ 16 of the specification expressly contemplates the use of other agents, specifically antibodies. Further, as reflected in the attached publications in Exhibit A, antibodies to clusterin were known as the of the filing date of this application. Applicants note that the Examiner states that "written description requires more than a mere statement that something is part of the invention." (Page 4) Applicants would appreciate an explanation of what this "more" is, and the legal basis for the requirement.

The Examiner rejected claims 1-3, 6-8 and 11-13 under 35 USC § 112, second paragraph as indefinite, stating that the meaning of the term "non-cancerous angiogenesis-related disease" is unclear. Applicants have considered the Examiner's arguments, and have amended claims 1, 6 and 11 to indicate that the angiogenesis-related disease is one in which reduction of angiogenesis is desirable, and that reduction in angiogenesis occurs. Further, claims 16-18 have been added reciting specific diseases taken from Table 1 in the application. This list as set forth in the claim does not include atherosclerosis and hence the arguments based on Monia are not applicable.

The Examiner rejected claims 1, 2, 6, 7, 11 and 12 under 35 USC § 102(b) as anticipated by Monia based on the statement (without any supporting evidence) that antisense-targeted to clusterin can be used in treatment of atherosclerosis. Because atherosclerosis can be associated with increased angiogenesis, the Examiner argues that any treatment (or even a proposed treatment) must have resulted in a reduction of angiogenesis. Applicants submit that this argument is scientifically inaccurate. If the record established that angiogenesis were always involved atherosclerosis, and that atherosclerosis cannot occur in the absence of angiogenesis,

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then the Examiner's position (which amounts to an assertion of inherency) might have merit. However, such a showing has not been made by the Examiner, and inherency cannot be established in the absence of such certainty. *Ex parte Levy*, 17 USPQ2d 1461, 1464 (BPAI 1990). "Inherency . . . may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient." *In re Robertson*, 49 USPQ2d 1949, 1951 (Fed. Cir. 1999).

Applicants further note that clusterin, also known as apolipoprotein J, was known to form a high density lipoprotein complex with apolipoproteins A-I in human plasma. (See Exhibit B). Clusterin was also known to play a role in lipid transport and lipid redistribution. These activities have nothing to do with angiogenesis. Thus, the Examiner has not met the burden of showing that reduction of angiogenesis would necessarily have occurred if the teaching of the prior art were followed and the rejection is in error.

In response to the arguments submitted in the Appeal Brief, the Examiner has stated that nothing in the decision in Eaton Corp. v. Rockwell "precludes a finding of inherency." (Page 10). This may be true, but the Examiner has not made the showing of facts necessary to support a finding of inherency.

With respect to claim 6, the Examiner asserts that "Applicants' argument that one cannot infer that treatment of atherosclerosis using clusterin reduction would result in reduction of angiogenesis or that any therapeutic benefit that might flow from such a treatment would have anything to do with angiogenesis would appear to support an enablement rejection" seems rather a threat, but does not respond to the argument. The art says what it says, and it does not say anything about angiogenesis. Therefore the basis for a rejection that the Examiner admits to be grounded in inherency is not found in the reference. Furthermore, while economy of prosecution would suggest that a rejection that is appropriate should be made, not threatened, Applicants submit that there is no basis for an enablement rejection. Enablement is based on this specification, not on the failure of the art to recognize Applicants discovery and invention.

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Finally, the Examiner states that "Monia may have thought that a previously known activity ... is providing the effect of treating atherosclerosis, this does not preclude an inherent anticipation of the instant claims." (Page 12). Again, what Monia "may have thought" is not relevant, only what Monia actually says in the document relied upon is relevant to a rejection based on inherency. The sum total of this statement is that:

Clusterin is overexpressed in many disease states including neurodegenerative disorders, gliomas, retinitis pigmentosa and expression is induced in acute and chronic models of renal injury and disease, following ureter obstruction, ischemia/reperfusion, and atherosclerosis reviewed in (Silkenssen et al., Biochem. Cell. Biol., 1994, 72, 483-488). The pharmacological modulation of clusterin activity and/or expression may therefore be an appropriate point of therapeutic intervention in pathological conditions.

Monia thus discloses no testing and no actual activity of clusterin "modulation", and nothing upon which the Examiner can base her speculations.

For these reasons, the rejection for anticipation should be withdrawn.

The Examiner also rejects claims 1-3, 6-8 and 11-13 as obvious over the combination of Monia and Gleave. Gleave discloses the specifically claimed antisense sequences, but it silent with respect to non-cancerous conditions and angiogenesis. Accordingly, this rejection depends on the Monia for the teaching of atherosclerosis, and the extension that this inherently is a teaching relevant to angiogenesis. Thus, this rejection should be withdrawn for the same reasons as the anticipation rejection.

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For these reasons, this application is now considered to be in condition for allowance and such action is earnestly solicited.

Respectfully submitted,



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## Immunolocalization of Clusterin in the Ram Testis, Rete Testis, and Excurrent Ducts<sup>1</sup>

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### ABSTRACT

Clusterin, a glycoprotein that elicits cell aggregation, has previously been isolated from ram rete testis fluid, and has been partially characterized. In experiments reported, we have used monoclonal antibodies against clusterin in combination with indirect immunofluorescence microscopy to investigate the distribution of clusterin in the adult ram testis, rete testis, and excurrent ducts. Tissue blocks (5 mm<sup>3</sup>) were fixed in periodate/lysine/paraformaldehyde containing 0.1% glutaraldehyde and, after embedding, 5-μm sections were prepared for immunolocalization. In the testis, 2 basic patterns were observed: 1) strong to moderate staining for clusterin in the adluminal region with little staining in the basal region of the seminiferous epithelium and germinal cells; and 2) moderate staining throughout the seminiferous epithelium between germinal cells. In the rete testis, strong clusterin staining was localized intracellularly in the rete epithelial cells, most often associated with the luminal surface. In the epididymis, intracellular clusterin was localized in some principal cells of the caput epididymidis. The luminal surfaces and spermatozoa within the lumen were strongly positive. In the vas deferens, clusterin staining was associated with the luminal surface only. The presence of clusterin was clearly detected in unwashed isolated epididymal spermatozoa, but not in spermatozoa washed with phosphate-buffered saline containing 0.05% Tween 20.

### INTRODUCTION

It is generally accepted that cell interactions play an essential role in the development and cytodifferentiation of the seminiferous epithelium (Fawcett, 1975; Roosen-Runge, 1977). Nevertheless, the molecule(s) implicated in germ cell-somatic cell interactions are at present unknown. Rete testis fluid may contain molecules generated by one or more types of testicular cells that could be involved in cell interactions in the seminiferous tubule (Voglmayr et al., 1967; Blaschuk et al., 1983; Fritz et al., 1983). We have recently isolated and characterized a glycoprotein, clusterin, from ram testis fluid that elicits clustering of several types of cells (Blaschuk et al., 1983; Fritz et al., 1983). In this communication, we report the immunohistochemical localization of clusterin in cells of the testis, rete testis, and excurrent ducts.

### MATERIALS AND METHODS

#### Preparation of Tissue Sections

Testes, epididymides, and vasa deferentia of young adult rams (Suffolk breed) were obtained from a local abattoir in February and March and were transported on ice. Within 30 min the organs were dissected on ice, and 5-mm<sup>3</sup> tissue blocks were fixed by immersion in 4°C periodate/lysine/paraformaldehyde (PLP; McClean and Nakane, 1974) containing 0.1% glutaraldehyde. After fixation at 4°C for 24 h, the tissues were thoroughly washed in phosphate-buffered saline (PBS), dehydrated in graded ethanol, and embedded in Paraplast (Sherwood Medical Ind., St. Louis, MO). Sections of 5-μm thickness were prepared for immunostaining.

#### Isolation and Processing of Spermatozoa

Spermatozoa were isolated from epididymides of adult rams according to procedures previously described (Tung and Fritz, 1978). Briefly, the epididymides were diced into 1-2-mm blocks in PBS and agitated gently using a plastic pipette with an open-tip diameter of 5 mm. Spermatozoa released were separated from the epididymal fragments by filtering through 100-μm mesh stainless steel grids, and by washing (twice) with cold PBS, followed by centrifugation at 1000 × g for 10 min. Resuspended cells were adjusted to 1 × 10<sup>6</sup> cells/ml and 10-μl samples were mounted on microscope slides. Slides were air-dried for 15 min at room temperature, after which they were fixed either with: 1) PLP containing 0.1% glutaraldehyde for 30 min,

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<sup>2</sup>Reprint requests.

followed by immersion in methanol at -20°C for 7 min; or 2) with PLP-glutaraldehyde alone. Some slides were left unfixed. All slides were then washed in PBS, and the preparations were either used immediately or stored at -20°C for less than 30 days before examination.

For bulk adsorption, isolated spermatozoa were pelleted and fixed with PLP-glutaraldehyde, followed by washing with PBS. Adsorption was carried out as previously described (Tung et al., 1984). Spermatozoa preparations isolated from epididymis were examined for immunostaining before and after being washed further with PBS containing 0.05% Tween 20 (Bio-Rad, Richmond, CA) (PBS-Tween). Unfixed spermatozoa, accommodated in (15-mL) screw-capped centrifuge tubes, were resuspended in PBS-Tween at 4°C. The tubes were agitated at 4°C for 2 h by rotation at 10 revolutions per min. Spermatozoa were washed three times in PBS without Tween 20, resuspended in PBS, smeared, and fixed as described above.

#### *Monoclonal Antibodies*

Lymphocytes prepared from spleens and inguinal lymph nodes of Balb/c female mice previously immunized against partially purified ram clusterin (Blaschuk et al., 1983) were fused with Sp 2/0-Ag 14 myeloma cells. Hybridomas were grown in hypoxanthine/aminopterin/thymidine medium (OJ and Herszenberg, 1980) for 2 wk, after which they were propagated in hypoxanthine/aminopterin medium. Hybridomas generating immunoglobulin were expanded into 24-well Linbro plates, and assessed using an enzyme-linked immunosorbent assay (ELISA) as previously described (Fritz et al., 1985). Hybridomas that secreted immunoglobulin G<sub>1</sub> (IgG<sub>1</sub>) against clusterin were cloned by limiting dilution in 96 well plates. Clones were propagated in RPMI 1640 culture medium containing 10% fetal bovine serum, and were used for the production of ascites fluid in female Balb/c mice (Fritz et al., 1985). Immunoglobulin G<sub>1</sub> was purified from ascites fluid by ammonium sulfate fractionation, and ion exchange chromatography on DEAE-Sephadex CL-6B (Pharmacia, Quebec, Canada) equilibrated with 20 mM potassium phosphate (pH 8.0) (Good et al., 1980). In experiments reported in this communication, anti-clusterin antibody preparations employed were those secreted by a hybridoma named HCA 17 (Blaschuk et al., 1983; Fritz et al., 1985).

#### *Immunoistochemistry*

Immediately before immunostaining, tissue sections were deparaffinized and hydrated. Tissue sections or

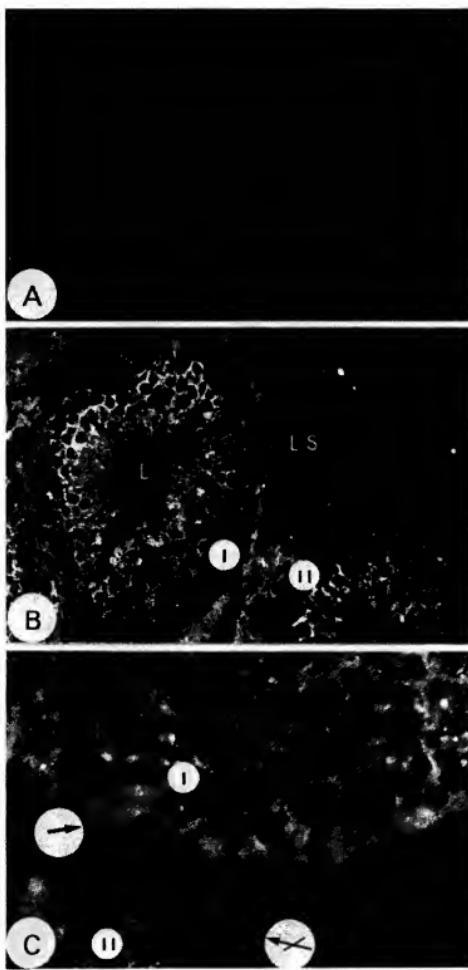
smears were incubated in 1% normal sheep serum in PBS for 30 min at room temperature to block non-specific staining. The sections were blotted with filter paper, followed by incubation for 30 min with specific monoclonal antibodies at various concentrations in PBS. Sections were washed in PBS-Tween, after which affinity-purified F(ab')<sub>2</sub> fragments of sheep antimouse IgG conjugated with fluorescein isothiocyanate (FITC; Cappel Lab., West Chester, PA), diluted 1:10 with PBS, were added. Sections were incubated for another 25 min, followed by additional washing with PBS-Tween. Preparations were mounted in 0.1 M *n*-propyl gallate (Sigma, St. Louis, MO) + glycerol (Giboh and Sedat, 1982), and examined by fluorescent microscopy as previously described (Tung and Fritz, 1978). Controls for the immunofluorescence studies included: 1) monoclonal antibody (IgG<sub>1</sub>) against rodent histocompatibility antigen IA<sup>K</sup>, which had been demonstrated in pilot experiments to be negative to ram tissues, in place of antibody against clusterin; 2) monoclonal antibody (IgG<sub>1</sub>) against clusterin preabsorbed with lyophilized ram testis fluid or spermatozoa isolated from ram epididymis (Tung and Fritz, 1984); and 3) PBS alone, followed by FITC-conjugated sheep antimouse IgG. All of these controls showed no specific staining.

#### RESULTS

##### *The Testis*

By indirect immunofluorescence microscopic procedures, material reactive with monoclonal antibody against clusterin was detected in the seminiferous epithelium (Fig. 1). In contrast, the luminal cavity, the boundary tissue of the seminiferous tubule, the interstitial tissues, the lymphatic spaces, the testicular capsule, and blood vessels were either negative or very weakly immunoreactive to monoclonal antibody staining (Fig. 1). Within the seminiferous tubule, two types of staining patterns were consistently observed: 1) strong to moderate staining in the seminiferous epithelium adjacent to the lumen, with very little staining in the basal region and spherical germinal cells (Fig. 1B); and 2) moderate immunostaining throughout the seminiferous epithelium between the spherical germinal cells (Fig. 1C). Sections

FIG. 1. Indirect immunofluorescence micrographs showing distribution of clusterin in the adult ram testis. A: A control section stained with monoclonal IA<sup>K</sup> antibody (0.1 mg/ml). B and C: Sections stained with monoclonal clusterin antibody (0.1 mg/ml). Note moderate to intense immunostaining of the seminiferous epithelium between the spherical germinal cells in both B and C. B shows staining in borders of nearly all cells in the adluminal region, and no staining in most cells in the basal region of the tubule. C shows both extracellular and intracellular staining with similar intensity throughout the adluminal and basal regions of the epithelium. The lymphatic space (LS) and lumen (L) are consistently unstained. Peritubular boundary tissues (arrow) and interstitial tissues (crossarrow) are weakly or negatively stained. Different tubules are labeled I and II for orientation. Magnifications: A, B  $\times 160$ ; C  $\times 630$ .



incubated with the monoclonal antibody against IA<sup>k</sup>, or with PBS were consistently not stained (Fig. 1A). Adsorption of the monoclonal antibody against clusterin with nonwashed epididymal spermatozoa fixed with PLP-glutaraldehyde, or with ram rete testis fluid, also reduced the staining intensities to negligible levels (data not shown).

#### The Rete Testis

In the rete testis, distribution of material reactive with antibodies against clusterin was characterized by strong intracellular staining between nuclei of both columnar and cuboidal epithelial cells (Fig. 2B-D). The luminal surface was often stained, but the luminal cavity and the connective tissues (mediastinum) underlying the epithelium were not stained.

#### The Epididymis

Throughout the entire epididymis, material strongly immunoreactive with monoclonal antibody against clusterin was associated with spermatozoa located within the lumen (Fig. 3A-D). The staining intensity decreased from caput (Fig. 3A,B) to cauda (Fig. 3D) epididymidis but was still detectable in the cauda epididymidis (Fig. 3D). Intracellular staining of the epithelium was observed only in isolated areas of the caput epididymidis where diffuse immunoreactive material was localized in both basal and supranuclear regions of principal cells (Fig. 3A,B). The luminal surfaces of caput, corpus, and cauda epididymidis were strongly immunoreactive to monoclonal clusterin antibody (Fig. 3A-D). The interstitium underlying the epididymal cells was not stained (Fig. 3D).

#### The Vas Deferens

Immunostaining for clusterin was detected in moderate to weak intensities on the luminal surfaces of the vas deferens, and also in unidentified particles in the lumen. No specific intracellular staining was found within the epithelium, nor in the underlying connective tissues and smooth muscle layers (Fig. 4A).

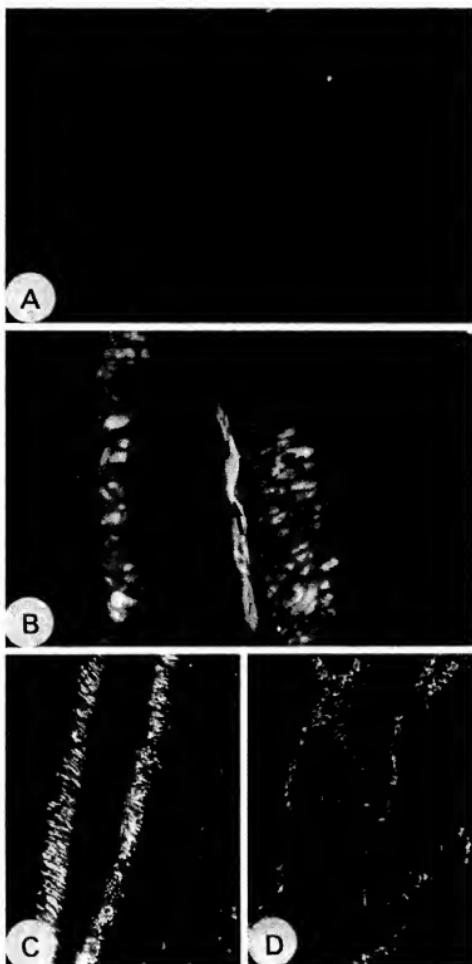
#### The Spermatozoa

Spermatozoa isolated from ram epididymis specifically stained with the monoclonal antibody against clusterin. Fixation by PLP-glutaraldehyde, with or without permeabilization by methanol, exhibited identical patterns of immunostaining, suggesting that clusterin was associated exclusively with the surface of the spermatozoa. The immunostaining pattern was characterized by a strong to moderate staining intensity on the sperm head, with a punctate appearance. In contrast, the tail was uniformly stained (Fig. 4B). Washing of sperm with PBS-Tween prior to fixation totally removed immunostaining (Fig. 4C), suggesting that clusterin may be nonspecifically bound to spermatozoa.

#### DISCUSSION

We have reported the immunohistochemical distribution of clusterin in the testis, rete testis, epididymis, vas deferens, and isolated spermatozoa of the ram. Data presented indicate that immunoreactive clusterin can be localized *in situ* in the seminiferous tubule, with a distribution characteristic of that associated with Sertoli cells. These data are consonant with our previous observations that clusterin can be synthesized by Sertoli cell-enriched preparations in culture (Blaschuk et al., 1983; Blaschuk and Fritz, 1984). In addition, the present paper also demonstrates the apparent intracellular localization of clusterin in rete epithelial cells and in some principal cells of the caput epididymidis. This localization is of particular interest in light of previous findings that rete epithelial cells lack ultrastructural features characteristic of secretory cells (Dym, 1976). Furthermore, we have demonstrated in this paper that clusterin is loosely associated with spermatozoa found in the epididymis and vas deferens, and with the luminal surfaces throughout the epididymis and vas deferens. This is consistent with previous observations that clusterin is present in significant concentrations in epididymal fluids (Fritz et al., 1985). Clusterin was readily removed

FIG. 2. Indirect immunofluorescence micrographs showing distribution of clusterin in the rete testis of an adult ram. A: A control section stained with monoclonal IA<sup>k</sup> antibody (0.05 mg/ml). B,C, and D: Sections stained with 0.05 mg/ml monoclonal antibody against clusterin. Note intracellular distribution of immunostaining in columnar cells (B,C), and cuboidal cells (D) in the rete epithelium. Portions of the luminal surface (arrow) are also stained (B), whereas the mediastinum surrounding the lumen is weakly or negatively stained. Magnifications: A,B X630; C,D X160.



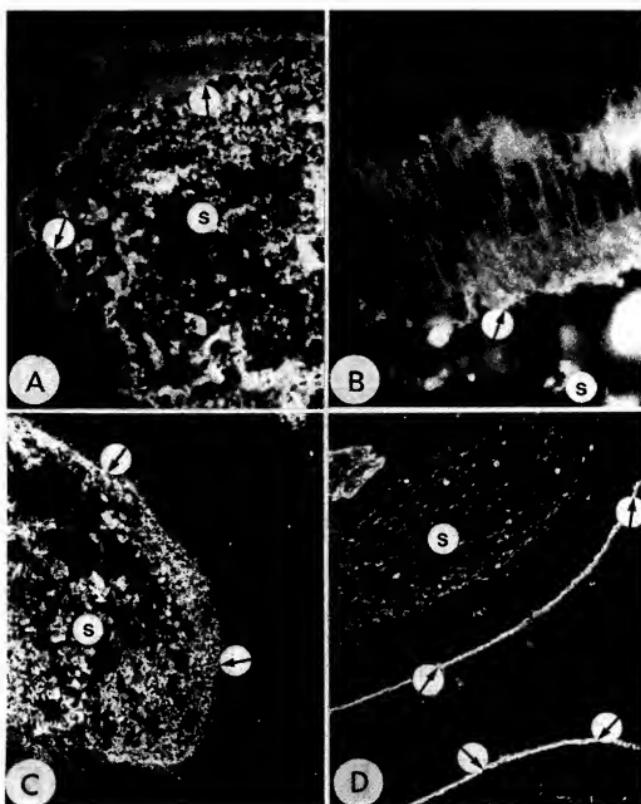


FIG. 3. Indirect immunofluorescence micrographs showing distribution of clusterin in the caput (A and B), corpus (C), and cauda epididymidis (D). Note occasional intracellular immunostaining principal cells of the caput epididymidis (A and B), but negative intracellular staining in epithelial cells of the corpus and cauda epididymidis (C and D). The spermatozoa (s) in the lumen and the luminal surfaces (arrows) are consistently positive throughout the epididymis, but the connective tissues underlying the epithelium are unstained. Magnifications: A, C, and D X160; B X630.

from unfixed sperm by washing (Fig. 4), but remained bound to sperm in fixed tissue sections (Fig. 3). We interpret these data to indicate that clusterin is nonspecifically bound to spermatozoa, but that it can be cross-linked to sperm surfaces by treatment with glutaraldehyde.

In the mature testis, two basic (adluminal and panepithelial) patterns of distribution of clusterin were observed within the seminiferous tubule. The variations in the distribution of clusterin might be dependent on the stage of the cycle of the seminiferous epithelium, a phenomenon known to occur in the localization of androgen-binding protein (Rizzen et al., 1980; Attramadal et al., 1981) and in the distribution of plasminogen activator (Lacroix et al., 1981; Fritz and Karmaly, 1983). In the mature testis, the tight junctional complexes among Sertoli cells limit the transport of fluids and macromolecules between the basal and adluminal compartments (Fawcett, 1975; Setchell and Waites, 1975). Clusterin is probably secreted by Sertoli cells primarily into the adluminal portion of the seminiferous epithelium, released into the tubular lumen, and then transported into the rete testis. In some sections, clusterin was also detected in the basal region of the epithelium. Relatively small quantities could have been released into the lymphatics, accounting for the clusterin present in testicular lymph (Fritz et al., 1985). Androgen-binding protein release into the basal compartment is also known to occur (Bardin et al., 1981). The blood-testis barrier has been shown to be structurally (Dym, 1976) and functionally (Tung et al., 1971) incomplete in the rete testis. Nevertheless, most protein molecules do not seem to escape from the plasma across the rete epithelium into the lumen (Setchell and Waites, 1975; Dym, 1976). It remains possible that small quantities of clusterin could have been released from the basal surfaces of rete epithelial cells through the poorly vascularized mediastinum (Kormano and Suoranta, 1971; Dhingra, 1977) into the testicular lymph, especially if the rete testis epithelial cells synthesize clusterin.

The intracellular localization of clusterin in rete epithelial cells indicates a cell-specific distribution within the tissue. The data indicate that rete cells may be capable of clusterin biosynthesis, or that they absorb clusterin from the rete testis fluid. Absorption by rete testis epithelial cells seems to be minimal, as judged by the observations that Thorotrast injected

into the lumen of hamster and rat rete testes is rarely detected within rete epithelial cells (Dym, 1976). Although lacking the ultrastructure suggestive of active secretory functions (Dym, 1976), rete epithelial cells have been demonstrated to possess secretory activities (Renzali, 1957; Goyal and Dhingra, 1973; Setchell, 1974). Using cultures of enriched preparations of ram rete testis cells for immunocytochemical localization and immunoassay, we have recently observed that rete testis cells synthesize clusterin to an extent comparable to that detected in Sertoli cell-enriched preparations in culture (Tung and Fritz, in preparation). We have previously reported that the concentrations of clusterin in epididymal fluid (30–120 µg/ml) are only moderately lower than those in rete testis fluid (80–200 µg/ml) (Fritz et al., 1985). By contrast, concentrations of clusterin in testicular lymph (4–16 µg/ml) and systemic plasma (0.8–3.2 µg/ml) are very low in comparison to those in rete testis or epididymal fluids (Fritz et al., 1985). These observations suggest that clusterin is poorly resorbed by epithelial cells throughout the rete and the excurrent ducts. We interpret available data to indicate that immunoreactive clusterin localized intracellularly in rete epithelial cells represents active *in situ* biosynthesis rather than resorption of this molecule. The data also suggest that clusterin in rete testis fluid is derived from rete epithelial cells as well as from Sertoli cells. The precise amounts contributed by each class of cells *in vivo* remain to be determined.

Clusterin induces marked aggregation of several cell types *in vitro* (Blaschuk et al., 1983; Fritz et al., 1983). Its possible role in cell-cell interactions in the testis has previously been postulated (Fritz et al., 1983; Blaschuk and Fritz, 1984), but it has not been ruled out that high levels of clusterin within the rete and the excurrent ducts may have functions other than those involved in cell cohesion. For example, clusterin could be a candidate among molecules uniquely present in high concentrations in testicular and epididymal fluids that play a role in providing immunologic privilege to germinal cells. It is generally accepted that the seminiferous tubule barrier (often erroneously called the blood-testis barrier) protects germinal cells in the adluminal compartment from the immunologic surveillance system (Dym and Fawcett, 1970; Johnson, 1973; Gilula et al., 1976; Neaves, 1977). However, other factors may be involved in shielding the germinal cells

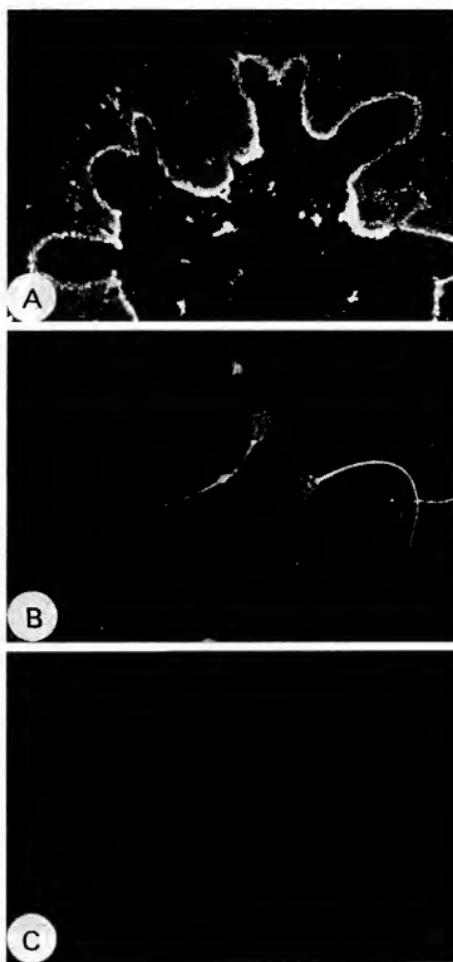


FIG. 4. Localization of clusterin by indirect immunofluorescence in the ram vas deferens (A); spermatozoa isolated from the epididymis and washed with PBS (B); and epididymal spermatozoa washed with PBS-Tween (C). Note strong immunostaining associated with the luminal surface and residues in the vas lumen (A) and with epididymal spermatozoa washed only with PBS (B), and the total removal of specific staining from spermatozoa washed with PBS-Tween (C). Magnifications: A  $\times 160$ ; B and C  $\times 640$ .

from potentially deleterious immunoglobulins and self-reactive lymphocytes (Tung and Fritz, 1984), especially in the rete testis, the epididymis, and the vas deferens duct, in which the barrier is functionally incomplete in excluding spermatozoa from immunoreactants (Korman, 1967; Koskinies et al., 1971; Tung et al., 1971; Johnson, 1972; Koskinies and Korman, 1973; Suzuki and Nagano, 1978). Lymphocytes are present among the resident epithelial cells of the rete testis and epididymis (Dym and Romrell, 1975), suggesting that the potentially immunogenic sperm cells in the lumen are indeed under immunosurveillance. It therefore seems plausible to postulate the presence of components in these portions of the male reproductive tract that act as local immunosuppressants. Experiments are in progress to determine if clusterin may have local immunosuppressing activity, perhaps by blocking recognition by lymphocytes of germinal cells as "foreign."

#### ACKNOWLEDGMENTS

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## Immunohistochemical Localization of Clusterin in the Male Genital Tract in Humans and Marmosets

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### ABSTRACT

Clusterin is a multifunctional protein, first described in the reproductive tracts of the rat and the ram. It is produced by several cell types and exists in at least two differentially glycosylated forms. The aim of this study was to extend knowledge of clusterin expression in the primate (human and marmoset) male reproductive tracts by means of clusterin-specific immunohistochemical techniques. In both normal and abnormal testicular tissue, clusterin was found in association with Sertoli cells, luminal sperm, proacrosomal Golgi complexes, residual bodies, and degenerating germ cells. The major differences observed between the two groups were attributable primarily to morphological differences rather than to clusterin expression specifically. There was no correlation between testicular clusterin content and the cause and severity of spermatogenic disorders. Within normal epididymides, regional differences in clusterin staining similar to those reported in the rat were observed. The seminal vesicles contained large amounts of positive clusterin staining, whereas normal human prostate was completely negative. Low levels of clusterin expression were observed in the marmoset prostate. This study suggests that clusterin is an important and widespread product in the human and marmoset reproductive tracts and is likely to have a role in spermatogenesis.

### INTRODUCTION

A glycoprotein, now generically referred to as clusterin, has recently been identified in the blood, seminal plasma, and several solid tissues of many species. Clusterin was first identified in the male reproductive tracts of the rat and ram, where it was named sulphated glycoprotein-2 (SGP-2) and clusterin, respectively [1-3]. It was subsequently found in several other species and has been implicated in a number of processes including lipid transport, complement inhibition, secretory processes, and programmed cell death. The name clusterin was proposed because of the ability of this protein to aggregate homologous cell types *in vitro* [4]. Clusterin has also been found to be a major component of Sertoli and epididymal cell secretions both *in vitro* and *in vivo* [1, 5]. Multiple functions have been proposed for clusterin on the basis of its biochemical and structural properties.

The aim of the present investigation was to further characterize the sites of clusterin production in the normal and pathological human male reproductive tract through use of clusterin-specific immunohistochemical techniques. For comparative purposes and because some normal human tissues were not available, material from a primate, the marmoset, was also studied.

### MATERIALS AND METHODS

#### Tissues and Fixation

Portions of both spermatogenically normal ( $n = 16$ ) and pathological ( $n = 16$ ) human testes were obtained, by either

needle or open biopsy, from men undergoing investigation of infertility at The Royal Women's Hospital (Melbourne, Victoria, Australia). Fine-needle tissue aspiration biopsies were performed under local anesthesia with a modified 20-gauge, 5-cm Menghini or Turner type needle [6]. The testicular tissue was placed in heparinized minimum essential culture medium (MEM; Commonwealth Serum Laboratories, Melbourne, Victoria, Australia) to prevent clotting of any blood or hydrocele fluid. Tissue was then transferred to a microcentrifuge tube (Eppendorf, Hamburg, Germany) containing a 5% agar plug (Serva, Heidelberg, Germany) in its base. The MEM was removed via a Pasteur pipette and the tube was filled with mercuric chloride fixative. Tissue was allowed to fix for 30 min, after which the tube was centrifuged at  $320 \times g$  for 30 min so that the tissue would settle onto the agar plug. Tissue was then overlaid with 7  $\mu\text{l}$  of eosin Y (1% eosin:95% ethanol) followed by another layer of agar. The agar plug was removed from the Eppendorf tube, trimmed close to the interface of its two layers (as marked by the eosin), and embedded in paraffin by standard procedures. Open testicular biopsies were performed at The Royal Women's Hospital at the time of surgical procedures that included vasovasostomy, vasoperitoneal, and microsurgical epididymal sperm aspiration. Abnormal testicular biopsies included those with the histopathological diagnoses of severe ( $n = 3$ ), mild ( $n = 7$ ), and moderate ( $n = 3$ ) hypospermatogenesis [7]; Sertoli cell-only syndrome ( $n = 2$ ), and Leydig cell adenoma ( $n = 1$ ).

Samples of pathological epididymis were obtained from three men undergoing sperm collection for *in vitro* fertilization because of congenital absence of the vas deferens. Epididymal tissue was treated in the same way as testicular material.

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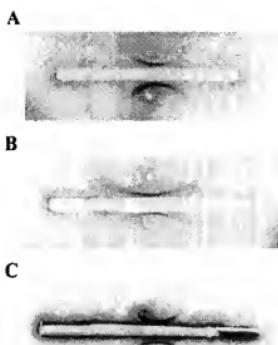


FIG. 1. Immunoelectrophoresis gels showing the monospecificity of the anti-human clusterin antiserum diffused against (A) human blood serum, (B) human seminal plasma, and (C) marmoset blood serum. The single precipitin arcs indicate that the antiserum is monospecific.

Histologically normal human seminal vesicles ( $n = 2$ ) and prostates ( $n = 2$ ) were obtained from autopsy material (St. Vincent's Hospital, Melbourne, Victoria, Australia). Abnormal prostatic tissue was also collected during surgery for benign prostatic hypertrophy and was fixed in either mercuric chloride or in 10% neutral buffered formalin. Prostatic tissue was classified as morphologically normal or hypertrophic by an independent pathologist with no prior knowledge of the results of immunohistochemistry. Abnormal tissues showed mixed ductal and glandular hypertrophy. Clusterin was localized in mercuric chloride-fixed tissue using either a monoclonal antibody or a polyclonal anti-human clusterin antiserum as the primary antibody as described below. Formalin-fixed tissue was stained by means of the polyclonal antiserum.

Nothuman primate tissue was obtained from common marmosets (*Callithrix jacchus*), as we had ready access to tissues from animals killed by an overdose of barbiturates for other purposes. Normal adult marmoset tissues were obtained by Dr. A. Lopata ( $n = 2$ ; The Royal Women's Hospital) and by Ms. J. Clarke ( $n = 4$ ; CSIRO, Division of Nutrition, Adelaide, Victoria, Australia). The marmosets were mature and of different ages, and they had not been used for breeding. Reproductive tract tissues collected included testes, epididymides, seminal vesicles, prostates, and vas deferentia. All tissues were removed immediately after death and fixed in mercuric chloride fixative for 4 h.

For immunohistochemistry, 3- $\mu\text{m}$ -thick tissue sections were cut using a microtome, floated onto aminoalkylsilane-

treated or gelatin-coated slides, dried at 37°C overnight, and stored at room temperature until used.

Similar distributions of clusterin were obtained from pilot studies using both unfixed tissues and tissues fixed by a variety of methods; however, morphology and staining were of the highest quality with mercuric chloride-fixed and paraffin-mounted tissues. Trial fixation procedures included snap-freezing tissue without fixation; fixing frozen tissue with paraformaldehyde-lysine-periodate; and fixing paraffin-mounted tissues with Bouin's, 10% neutral buffered formalin, zinc formalin, 6% zinc sulphate, 4% paraformaldehyde, or mercuric chloride.

#### Antibodies

Immunohistochemical studies on human tissues were performed using the monoclonal antibody E5 described by Murphy [8]. The primary antibody used for immunohistochemistry on marmoset tissues was an anti-human clusterin polyclonal antiserum produced in sheep. The clusterin used as an immunogen to produce the anti-human clusterin antiserum was purified from human seminal plasma by affinity chromatography as described previously [9]. This purified clusterin was free of contaminating proteins as determined by SDS-PAGE and reacted strongly by Western blotting with anti-human clusterin monoclonal antibodies [8]. Sheep were immunized four times at monthly intervals with approximately 0.1 mg of the purified clusterin emulsified in Freund's incomplete adjuvant (Sigma, St. Louis, MO). Sheep were bled from the jugular vein and the serum was recovered. The immunoglobulin fraction was purified by means of ammonium sulphate precipitation [10]. An equal volume of ice-cold, saturated ammonium sulphate (BDH), pH 7.0, was added drop-wise to the serum and proteins were allowed to precipitate for 90 min at 0°C. The immunoglobulin was recovered by centrifugation at 5000 rpm for 10 min. The pellet was resuspended in 0.1 M PBS, pH 7.5 and dialyzed extensively. The antiserum was demonstrated to be monospecific by immunoelectrophoresis as described below and shown in Figure 1.

#### Immunobistochemistry

Prior to probing for clusterin, sections were dewaxed with xylene and rehydrated through graded ethanol into water. Clusterin localization was demonstrated through use of an avidin-biotin amplified immunoperoxidase technique. Human tissue sections were sequentially incubated with the E5 anti-clusterin monoclonal antibody, biotinylated anti-mouse immunoglobulins (1:400, RPN.1061; Amersham, Bucks, UK), and streptavidin-conjugated peroxidase (1:400, P397; Dako, Glostrup, Denmark). The localization of clusterin in marmoset tissues, and in human prostatic tissue fixed in 10% neutral buffered formalin, was demonstrated via a similar technique with the exception that anti-human clusterin polyclonal antiserum (1.5  $\mu\text{g}/\text{ml}$ ) was used as the

primary antibody and biotinylated anti-sheep/goat immunoglobulins (1:400, RPN1025; Amersham) were used as the second step. Bound peroxidase was detected on both human and marmoset tissues by use of 0.05% diaminobenzidine tetrahydrochloride (DAB; Sigma) in phosphate buffer for 4 min. Endogenous peroxidase activity and nonspecific antibody binding were blocked by preincubating slides in 0.45% hydrogen peroxide and 10% nonimmune serum (rabbit serum for human tissue and swine serum for marmoset tissue, DAKO), respectively. For negative control sections, an irrelevant monoclonal antibody of the same immunoglobulin subclass (IgG<sub>1</sub>) was used as the primary antibody on human tissues and preimmune sheep serum was used on marmoset tissues. All sections were counterstained with Harris's hematoxylin.

#### *Monospecificity of the Anti-Human Clusterin Antiserum*

The monospecificity of the anti-human clusterin antiserum was determined by means of immunoelectrophoresis. Five microliters of either human or marmoset serum (1 to 2 dilution) or human seminal plasma (1 to 20 dilution) was electrophoretically separated on a 1% agarose:0.1 M PBS gel plate. Samples were separated at 10 V/cm for 90 min in 63.7 mM sodium barbital/1.2 mM barbital/1.8 mM calcium lactate (pH 8.6) on a flat-bed electrophoresis tray (2117 Multiphor II electrophoresis unit; LKB Pharmacia, Melbourne, Australia) with cooling (Multitemp II thermostatic circulator; LKB Pharmacia). After separation, the central trough of the plate was filled with antiserum and allowed to diffuse outward and form precipitates for 48 h at 37°C in a humid environment. Soluble proteins were removed by extensive washing with 0.01 M Tris, pH 7.8 over several days. Precipitin arcs were visualized by Coomassie blue staining.

## RESULTS

#### *Monospecificity of Anti-Human Clusterin Antiserum*

The anti-human clusterin antiserum was shown to be monospecific in human and marmoset serum and in human seminal plasma, as indicated by a single precipitin arc after immunoelectrophoresis (Fig. 1). Monospecificity was also confirmed by radial immunodiffusion and Western blotting of serum and seminal plasma (data not shown).

#### *Normal Human Testis*

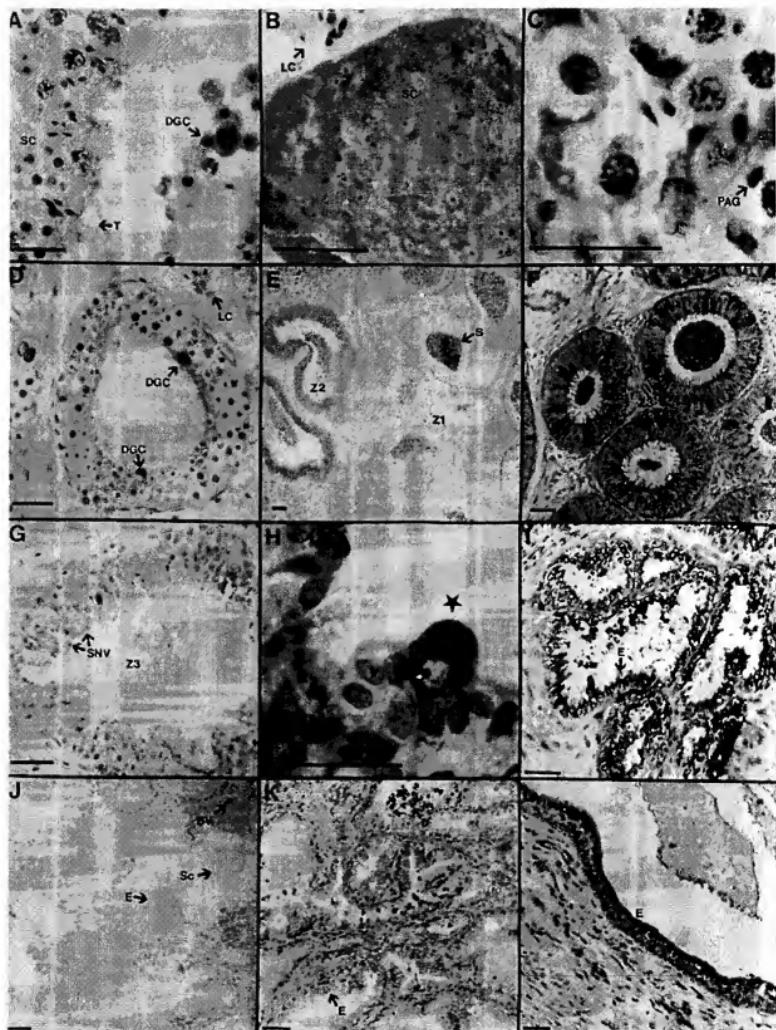
Within human testes with histologically normal spermatogenesis, clusterin was localized to five major areas: 1) the cytoplasm of Sertoli cells—a punctate granular pattern of clusterin staining was seen throughout the entire cytoplasm of Sertoli cells, enveloping all stages of spermatogenesis (Fig. 2A); 2) immature spermatozoa tails, in the lumen of the seminiferous tubules (Fig. 2A); 3) proacrosomal Golgi complexes of primary spermatocytes and some early

spermatids (Fig. 2C); 4) residual bodies in the luminal cytoplasm of Sertoli cells (data not shown); and 5) isolated germ cells. These isolated germ cells were generally immature cells sloughed off into the seminiferous tubule lumen (Fig. 2A), although individual, clusterin-positive germ cells were also occasionally seen within the germinal epithelium (Fig. 2D). Leydig cells and the surrounding connective tissue contained no immunoreactive clusterin (Fig. 2D). No differences were observed in the distribution of clusterin between open and needle testicular biopsies.

#### *Abnormal Human Testis*

The distribution of clusterin observed in the abnormal testicular tissues was generally the same as that observed in biopsies with normal spermatogenesis (Fig. 2, B and D). The major differences seen between the two groups were due primarily to morphological differences rather than clusterin staining specifically. There appeared to be no relationship between the pattern and density of clusterin

FIG. 2. Immunohistological distribution of clusterin in human and marmoset genital tract tissues. A) Normal human testes. Clusterin immunostaining (brown coloration) was distributed throughout the cytoplasm of Sertoli cells (SC), on the tails of immature spermatozoa (T) seen in the lumen of seminiferous tubules, and in potentially dying or damaged germ cells (DGC). B) A seminiferous tubule from an individual with Sertoli cell-only syndrome. The cytoplasm of Sertoli cells was strongly immunoreactive for clusterin in the absence of germ cells. C) A spermatogenically normal human testis, showing intense staining for clusterin in the proacrosomal granules (PAG) of primary spermatocytes and some early spermatids. The immunostaining surrounding spermatogenic cells is in the cytoplasm of Sertoli cells that encapsulate germ cells during spermatogenesis. D) A moderately hypospermatogenic human seminiferous tubule containing two intensely clusterin-immunoreactive germ cells. Clusterin expression within these cells may indicate the occurrence of programmed cell death or some type of repair mechanism. Leydig cells (LC) and the surrounding connective tissue contained no immunoreactive clusterin. E) A section of human epididymis from an individual with congenital absence of the vas. Within zone 1 (Z1), principal cells have clusterin-positive stereocilia and luminal sperm (S). In zone 2 (Z2), principal cells were relatively taller, with longer stereocilia and small punctate clusterin deposits distributed throughout their cytoplasm but concentrated toward the apical surfaces. F) Zone 3 (Z3) from a normal marmoset epididymis showing the characteristically mottled appearance of clusterin staining in principal cells. G) Zone 3 of a human epididymis from a patient with congenital absence of the vas. Principal cells were morphologically abnormal and often lacked stereocilia. The large clusterin-positive supranuclear vacuoles may represent accelerated endocytosis of tubular contents. H) A section of normal human seminal vesicle showing clusterin staining in principal cells. The marker cell (\*) was being extruded into the lumen of the gland via a holocrine process. Secretions within the gland were also stained for clusterin. I) A section of normal marmoset seminal vesicle. Similarly to human seminal vesicles, the marmoset seminal vesicles showed staining for clusterin within epithelial cells (E) and secretions; however, unlike the situation in the human gland, the marmoset gland also contained spermatozoa that were positively stained. J) A normal human prostate. Neither the epithelial cells (E) nor secretions (Sc) were reactive for clusterin. The presence of clusterin in blood vesicles (BV) acts as an internal positive control. K) A section of normal marmoset prostate showing low levels of clusterin within epithelial cells (E) and higher levels within circular structures (D) within the gland. L) Within the normal marmoset vas deferens, clusterin was localized predominantly to the epithelial cell layer (E). The underlying connective tissue and smooth muscle layers contained relatively little clusterin. Scale bars represent 50 µm.



staining and the type of spermatogenic disorder. The high density of clusterin staining in Sertoli cells from Sertoli cell-only testes (Fig. 2B) suggests that clusterin production may occur independently of germ cells.

#### *Marmoset Testicular Tissue*

In the marmoset testis (data not shown), the pattern of clusterin expression was the same as that observed for the samples from humans. Cytoplasm of Sertoli cells at all stages of spermatogenesis, luminal sperm tails, residual bodies, proacrosomal granules, and isolated (possibly damaged) germ cells were positively stained for clusterin.

#### *Marmoset Epididymal Tissue*

Within the marmoset epididymis, three distinct regions of tubular staining were observed and identified according to the morphological description of the normal rat epididymis [11]. Within the most proximal tubules (zone 1), the principal cells were relatively tall and possessed long, clusterin-positive stereocilia; the nuclei were basally located and the lumen contained large numbers of strongly clusterin-positive sperm. Morphologically and immunohistochemically this region corresponded with the distal initial segment described in the rat epididymis. The second region (zone 2), which corresponded to the intermediate zone in the rat, contained slightly taller principal cells; these, while similar in morphology to those in zone 1, exhibited longer stereocilia and had small punctate clusterin deposits distributed throughout their cytoplasm, but concentrated towards their apical surfaces (data not shown; however, similar to human tissue, Fig. 2E). Sperm within this region were also strongly stained for clusterin. Zone 3 of the marmoset epididymis contained tubules with a characteristically mottled appearance (Fig. 2F). Levels of clusterin in principal cells within this region varied markedly between adjacent cells; and the distribution, unlike that in regions 1 and 2, was evenly seen throughout the entire cytoplasm.

#### *Human Epididymal Tissue*

The human epididymal tissue studied was from men with a congenital absence of the vas deferens. As a result, the epididymal body and tail segments were absent and the lumen of the more distal caput segments was dilated and contained abnormal principal cells. Regions with morphological characteristics and clusterin distribution similar to those of regions 1 and 2 of the marmoset were present (Fig. 2E). The most distal of the epididymal regions (region 3), however, appeared abnormal in that it possessed principal cells varying in height and nuclear position and often lacking stereocilia. The most striking feature of this region was the presence of large clusterin-containing supranuclear vacuoles within the principal cells (Fig. 2G).

#### *Marmoset Rete Testis*

Small amounts of staining for clusterin were observed within epithelial cells of the marmoset rete testis (data not shown), mostly confined to the apical surfaces. It is possible that this may represent clusterin present within rete testis fluid.

#### *Human Prostate*

In normal human prostate, neither the secretions nor the epithelium contained immunoreactive clusterin (Fig. 2J). In the abnormal prostatic tissue, however, there was variation in the distribution of clusterin. Two of the three samples examined contained no clusterin staining; but in the other specimen, from a patient with benign prostatic hyperplasia, clusterin was localized to the hyperplastic epithelial cells and (to a lesser extent) to the underlying stromal cells in a small proportion of the glandular cross sections (data not shown). There was intense staining for clusterin in some epithelial cells, but the majority of tubules within this tissue were nonreactive for clusterin.

#### *Marmoset Prostate*

Normal marmoset prostatic tissue, unlike human tissue, contained low levels of clusterin. The epithelial cells of the prostatic collecting ducts showed a heterogenous expression of clusterin protein with a pronounced difference in clusterin levels between adjacent cells. Some cells appeared to contain large amounts of clusterin; others, negligible amounts. Higher levels of clusterin were observed in significant numbers of circular structures within the lumen of some regions of the gland (Fig. 2K). From the present study it is difficult to determine whether these structures were sloughed-off epithelial cells or the products of a secretory process. The majority of these structures did not appear to have nuclei.

#### *Human Seminal Vesicles*

Part of the seminal vesicle's secretions are produced by a holocrine process in which entire cells are sloughed off into the lumen. Many of these cells as well as the secretions in the lumen were stained strongly for clusterin (Fig. 2H).

#### *Marmoset Seminal Vesicles*

Within the seminal vesicles of the normal adult marmoset, clusterin was localized to epithelial cells and secretions (Fig. 2I) in a manner similar to that observed for the normal human seminal vesicles. Unlike the situation for the human tissue, marmoset seminal vesicles contained some spermatozoa within the lumen of the gland. These spermatozoa exhibited some clusterin staining on their tails.

#### *Marmoset Vas Deferens*

Within the vas deferens of marmosets, clusterin was localized to the epithelial cell layer (Fig. 2L). The underlying

stromal cells contained relatively little clusterin staining. Spermatozoa within the lumen of the vas deferens were stained for clusterin, particularly in the head region.

## DISCUSSION

In a number of recent studies, clusterin expression has been identified in a variety of cell types from many species: in the regressing ventral prostate of castrated rats (TRPM-2) [12, 13], in bovine adrenal medullary chromaffin granules (Gp-III) [14], as a component of high density lipoproteins in human serum (ApoJ) [15, 16], in canine kidney cell cultures (Gp80) [17], as a hippocampal mRNA elevated during neurodegenerative diseases [18], and as an avian sarcoma virus-induced product (T64) [19]. Human clusterin (SP-40,40) was first described by Murphy [8] in association with glomerular immune deposits and as a component of normal blood plasma and the soluble SC5b-9 terminal complement complex. It has subsequently been shown to be an inhibitor of reactive hemolysis and to be present in high concentration in human seminal plasma [9, 20, 21].

In the male rat reproductive tract, clusterin is produced by several different cell types and appears to be deposited on, and removed from, maturing spermatozoa in a manner that suggests multiple functions. Within rat testes, clusterin is found in the cytoplasm of Sertoli cells at all stages of spermatogenesis [22], in late residual bodies, and on the surface of luminal spermatozoa [23]. The production of clusterin by Sertoli cells has been shown to be independent of both testosterone and germ cells [24].

In the rat excurrent ductal system, however, clusterin appears to be removed from spermatozoa in the rete testis and efferent ducts before being recoated in the epididymis. Recent data suggest that within the rete testis and efferent ducts, clusterin is endocytosed from the testicular plasma by the epithelial cells and broken down in secondary lysosomes. As a consequence, sperm within the proximal initial segment lack immunoreactive clusterin [11, 25]. Principal cells from the distal initial segment and caput, corpus, and cauda epididymidis however, synthesize and secrete clusterin and thereby recoat the maturing spermatozoa [11]. The presence of two distinct types of clusterin in the rat (a testicular and an epididymal form) is supported by the results of Sylvester and coworkers [26], who have demonstrated glycosylation differences between clusterin isolated from testicular and epididymal sperm and an apparent absence of clusterin on spermatozoa from the efferent ducts. In the vas deferens of the rat, the surface of sperm appears to contain clusterin.

The distribution of clusterin within human tissues as presented in the current paper must be interpreted with some caution. Because of the difficulties in obtaining "normal" reproductive tract tissues, immunohistochemistry was of material obtained from patients undergoing treatment for fertility disorders or undergoing prostatic surgery, or was of autopsy material.

In the human male reproductive tract, as in that of the rat, clusterin is found at multiple sites, suggesting it may have a role in several different functions. The expression of clusterin in the Sertoli cell cytoplasm at all stages of spermatogenesis in both normal and pathological testicular biopsies indicates a role for clusterin that is not necessarily related to the presence or activity of spermatogenesis. In particular, the high levels of clusterin produced by Sertoli cells in Sertoli cell-only testes confirm the finding of Roberts [24] that clusterin synthesis and secretion may occur in the absence of germ cells.

Clusterin in spermatogenesis may function in a more general cellular role such as in the rearrangement of lipids or in the secretions of Sertoli cell products—similar to the previously proposed roles for clusterin in blood plasma as an apolipoprotein [15, 16] and in the adrenal gland in the recycling of secretory granules, respectively [14]. Such a general role would not be specific for the process of spermatogenesis and might therefore explain expression in the absence of spermatogenesis. It seems likely, however, although this is as yet unproved, that clusterin as a major human and marmoset Sertoli cell product is required for normal spermatogenesis.

Clusterin was also detected in the cytoplasm of occasional germ cells in both the human and marmoset testis. The majority of the clusterin-containing germ cells appeared abnormal histologically or were free in the lumen of the seminiferous tubules. It has been suggested that clusterin may be involved in the "programmed death process" in several other cell types, most notably the androgen-deprived prostate [12, 13]. However, it is not known whether this hypothesis also holds true for spermatogenic cells undergoing developmentally regulated cell death, although a large proportion of the germ cells produced by meiosis during spermatogenesis never leave the testes and are thought to die by the process of programmed cell death [27, 28]. The increased level of clusterin expression seen in sloughed-off epithelial cells in the prostate may also reflect the activity of programmed cell death.

The presence of clusterin in the proacrosomal granules of primary spermatocytes also suggests a role for clusterin in the formation of the acrosomal cap. This is supported by our observation of clusterin localized in the acrosomal region of mature ejaculated human sperm (unpublished data). The presence of clusterin within the Golgi of spermatids suggests that clusterin is produced by germ cells at this stage of spermatogenesis. The possibility does, however, remain that this clusterin has been produced by Sertoli cells and taken up by spermatids. This issue could be resolved by in situ hybridization studies.

Using immunohistochemistry and immunoelectron microscopy, Hermo et al. [11] demonstrated the presence of clusterin within the cuboidal epithelial cell lining of the rat rete testis and within the columnar cells of the efferent ducts. Immunostaining was localized primarily over the apical and

supranuclear regions. Clusterin was found in the endocytic apparatus, endosomes, multivesicular bodies, and acid phosphatase-positive dense secondary lysosomes, all of which have previously been shown to be involved in the endocytic pathway [29]. In contrast to previous data showing clusterin production by rat rete testis cells in culture [30,31], clusterin was absent from the secretory apparatus of rat rete testis and efferent duct epithelium *in vivo* [11]. Thus, *in vivo* it appears most likely that clusterin in the rat rete testis is produced in a proximal location (*i.e.*, in the testes). Sperm within the rat rete testis and efferent ducts contained no immunoreactive clusterin [11]. The electron microscopic localization of clusterin within the structures of the endocytic pathway suggests that clusterin may be endocytosed from the rat testicular plasma by the epithelial cells of the rete testis and efferent ducts and degraded within secondary lysosomes [11, 25]. Although data are limited for the human and marmoset rete testis, immunohistochemistry suggests that clusterin may be endocytosed from the testicular plasma in a manner analogous to that in the rat.

Clusterin staining in the caput, cauda, and corpus of the normal marmoset epididymis is characterized by a mottled immunohistochemical appearance. These data accord with findings in the rat by Hermo [11], who has suggested that the mottled appearance reflects nonsynchrony of clusterin synthesis and secretion, in which different groups of cells secrete at different intervals. Spermatozoa within these regions were strongly immunoreactive with the clusterin antisera.

Within the distal regions of the epididymis from patients with congenital absence of the vasa, however, the abnormal terminal principal cells contained relatively less clusterin and this was confined to supranuclear granules. It is possible that as a consequence of the epididymal blockage, principal cells within this region have a higher than usual endocytic activity in order to minimize the buildup of sperm within the lumen. The clusterin-positive supranuclear granules may represent endocytosis of luminal contents. Our data on clusterin expression in the excurrent ductal system in the primate reproductive tract are consistent with findings in the rat [11] in that they show differences in regional clusterin expression within both the human and marmoset epididymis. Such regional differences suggest that clusterin is produced in response to different stimuli in different regions of the epididymis; these regional differences are also supportive of the heterogeneous response of epididymal epithelial cells to androgen deprivation described by Cyr and Roibaire [32].

Within the accessory sex glands of the rat, clusterin expression has also been demonstrated. In the prostate of androgen-deficient rats, clusterin is expressed in a manner concordant with periods of programmed cell death [12, 13]. In the normal rat prostate, however, clusterin is constitutively expressed, at lower levels than after castration, only in the proximal prostate [33, 34]. Conflicting results have

been reported concerning the expression of clusterin by rat seminal vesicles [35].

In contrast to published data concerning clusterin production in the rat prostate [33, 34], neither the secretions nor the epithelium of normal human prostate seen in the present study appeared to contain clusterin. However, because of the difficulties in obtaining normal human prostatic tissue and the relatively elaborate structural organization of the human compared to the rat prostate [36, 37], the possibility does remain that a portion of the human prostate might have been constitutively producing clusterin but was not surveyed. The present result is, however, in concordance with a previous analysis of seminal plasma that showed no clusterin in the seminal plasma of men with a congenital absence of the vasa and seminal vesicles [9]. Seminal plasma from these individuals consists predominantly of prostatic secretions.

Staining for clusterin was observed in one abnormal human prostate sample; however, the significance of this finding is not yet known.

Within the prostate of the normal marmoset, low levels of clusterin staining were observed in secretory epithelial and ductal epithelial cells. Higher levels were observed in circular structures within the lumen of the gland and in occasional collecting ductal epithelial cells. It is not known whether these circular structures represent sloughed-off, possibly dying, epithelial cells or secreted structures such as prostasomes [38]. Similar to the situation with terminal portions of the epididymis, not all cells of the collecting ducts showed the clusterin protein, suggesting nonsynchrony of clusterin production between adjacent cells.

In human seminal plasma samples, clusterin was detected in all normal and pathological specimens with the exception of those from men with a congenital absence of the vasa and seminal vesicles. The presence of clusterin in seminal plasma from vasectomized individuals is consistent with an extratesticular site of production, likely to be the seminal vesicles [9]. In human seminal vesicles, both the epithelium and secretions were strongly stained for clusterin. This is in accord with our previous data in seminal plasma [9] and with the immunohistological findings in the rat [35]. Clusterin found in seminal plasma from vasectomized men is therefore produced by the seminal vesicles. It seems unlikely that clusterin produced at this point in the reproductive tract would be functioning in a maturation-related capacity because of its contact with sperm only during ejaculation; rather, as has been suggested previously [20], it may be acting in a protective manner, possibly against complement-mediated damage in the female genital tract or alternatively in a selection process against less viable spermatozoa.

In the primate reproductive tract, as in that of the rat, clusterin is an abundant protein with a widespread distribution. It is likely to be an important product of the Sertoli cell in all species and may have a role in germ cell devel-

opment. The finding of differential surface coating of sperm along the reproductive tract and the suggestion that the different forms of clusterin are coated at different sites are intriguing and as yet unexplained. The lipid-binding properties of clusterin [15, 16] have led to the speculation that clusterin expression may be involved in cell membrane remodeling during maturation [39], in interactions between spermatogenic and Sertoli cells, and also in response to cell injury. Whether the previously characterized property of clusterin as a complement inhibitor [9, 40] is significant in protection of sperm within the female genital tract [20] is also to be determined.

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## Clusterin (Complement Lysis Inhibitor) Forms a High Density Lipoprotein Complex with Apolipoprotein A-I in Human Plasma\*

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Clusterin/human complement lysis inhibitor (CLL) is incorporated stoichiometrically into the soluble terminal complement complex and inhibits the cytolytic reaction of purified complement components C5b-9 *in vitro*. Using an anti-clusterin affinity column, we found that an additional protein component with a molecular mass of 28-kDa co-purifies with clusterin from human plasma. We show by immunoblotting and amino acid sequencing that this component is apolipoprotein A-I (apoA-I). By using physiological salt buffers containing 0.5% Triton X-100, apoA-I is completely dissociated from clusterin bound to the antibody column. Free clusterin immobilized on the antibody-Sepharose selectively retains apoA-I from total human plasma. Delipidated apoA-I and to a lesser extent ultracentrifugation-purified high density lipoproteins (HDL) adsorbed to nitrocellulose also have a binding affinity for purified clusterin devoid of apoA-I. The isolated apoA-I-clusterin complex contains approximately 22% (w/w) lipids which are composed of 54% (mole/mol) total cholesterol (molar ratio of unesterified/esterified cholesterol, 0.58), 42% phospholipids, and 4% triglycerides. In agreement with the low lipid content, apoA-I-clusterin complexes are detected only in trace amounts in HDL fractions prepared by density ultracentrifugation. In free flow isoelectric focusing, the purified apoA-I-clusterin complex has the same mobility as the native clusterin complex in human plasma and is found in the slow-migrating HDL fraction of fasting plasma. Our data indicate that clusterin circulates in plasma as a HDL complex, which may serve not only as an inhibitor of the lytic terminal complement cascade, but also as a regulator of lipid transport and local lipid redistribution.

Human clusterin (synonymous names: SP-40,40; complement lysis inhibitor, or CLL; SGP-2; TRPM-2; apolipoprotein J)<sup>1</sup> is a 70-kDa glycoprotein found at concentrations of 50–100 µg/ml in normal human plasma and at about 10-fold higher concentrations in human seminal plasma (1–4). Initially, clusterin was identified in ram rate testis fluid as a major glycoprotein that mediates aggregation and clustering

of a variety of homologous and heterologous cells *in vitro* (5). The serum form of sheep clusterin, as well as the deglycosylated testicular clusterin, however, lacked the cell-aggregating activity (6). Human clusterin isolated from blood and seminal plasma as well as rat seminal plasma clusterin, which is identical to the Sertoli cell-derived sulfated glycoprotein 2 (SGP-2), showed the same cell-agglutinating activity *in vitro* (2, 7).

Recently, clusterin was shown to be an integral constituent of the nonlytic soluble terminal complement complex (1, 8) which is formed by S-protein/virocentin and the components of the lytic complex C5b, C6, C7, C8, and C9. A monoclonal antibody raised against a contaminating component in S-protein preparations (9, 10) identified clusterin as an additional constitutive component of the terminal complement complex (1). Similarly, monoclonal antibodies raised against immune deposits in the kidney of glomerulonephritis patients (11) were shown to bind to clusterin (8), which co-localized with terminal complement complexes in those patients (12). The complete protein structure of human clusterin (1, 3, 4) and rat clusterin (SGP-2) (7, 13) has been determined by cDNA cloning and partial protein sequences are known for sheep (6) and hamster homologues (14). The protein precursor is post-translationally processed into two cysteine-linked 35-kDa subunits. The amino-terminal 76 residues display the typical features of an α-helical coiled-coil domain (1) and are followed by 22 residues (position 77–98) showing homology to the cysteine-rich thrombospondin type I modules in terminal complement proteins (3, 15).

Clusterin has also been characterized independently as TRPM-2 as one of those gene transcripts which are highly upregulated in regressing and involuting tissues (16, 17). Clusterin is induced in the prostate (16, 17) and in the androgen-dependent Shionogi mouse mammary tumor after androgen withdrawal (18), in the kidney in response to a pressure insult, in embryonic interdigital tissues that undergo programmed cell death, and in a mouse bladder tumor during chemotherapeutic regression (19). Increased mRNA levels have been observed in scrapie-infected hamster brain and in the hippocampus of patients with Alzheimer's disease and Pick's disease (14). A closely related quail protein called T64, of which 46% amino acid residues are identical to mammalian

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<sup>1</sup>The abbreviations used are: CLL, complement lysis inhibitor; SGP-2, sulfated glycoprotein 2; TRPM-2, testosterone-repressed

prostate message 2; C5b-9, the terminal complement complex; C5, C6, C7, C8 and C9, the fifth, sixth, seventh, eighth, and ninth component, respectively, of the complement system; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; apo, apolipoprotein; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; ITP, isoelectric focusing; TLC, thin layer chromatography; HPTLC, high performance TLC.

clusterin sequences, is highly inducible in response to the activity of pp60<sup>Yw</sup>. The tissue distribution of T64 mRNA in the quail (20) appears to be strikingly similar to that in the rat species (13), indicating that the functions of clusterin are highly conserved even among evolutionary distant species.

While purifying clusterin from fresh human plasma by monoclonal antibody-affinity chromatography, we observed that clusterin was consistently associated with an additional protein of human plasma. A partial amino-terminal sequence of this co-purifying protein was identical to that of apoA-I. In this report we have studied the interaction between clusterin and apoA-I in human plasma and demonstrate that clusterin forms a distinct type of HDL particles that may be involved in lipoprotein metabolism or lipid redistribution.

#### EXPERIMENTAL PROCEDURES

**Purification of ApoA-I-Clusterin Complexes.**—Blood was obtained from healthy fasting volunteers. Cells and plasma were separated by low speed centrifugation at 4 °C in the presence of 10 mM EDTA. After separation, 1 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine were added to the plasma fraction. A monoclonal antibody column was prepared as described (1). 50 µl of fresh plasma was diluted with 1 volume of 10 mM Tris-HCl, 10 mM EDTA, 500 mM NaCl, pH 7.4, and loaded onto a 5-ml affinity column at 4 °C overnight. The column was washed extensively with 300 ml of 10 mM Tris-HCl, 10 mM EDTA, 500 mM NaCl, pH 7.4. The bound material was eluted with 0.2 M glycine, pH 2.5, by collecting 0.5-ml fractions which were immediately neutralized with a solution of Tris-HCl, pH 8.0. The eight fractions with the highest protein concentration were pooled and dialyzed against 150 mM NaCl, 10 mM Tris-HCl, pH 7.4 (TBS), containing 10 mM EDTA and stored thereafter at 4 °C. Protein concentrations were measured with the bicinchoninic acid protein assay (Pierce Chemical Co.) using BSA as a standard (21). The weight-to-weight ratio between purified clusterin and apoA-I protein was estimated by scanning the intensity of Coomassie Blue-stained apoA-I and clusterin bands.

**Amino-terminal Sequence Analysis.**—The proteins of affinity-purified clusterin preparations were separated by SDS-PAGE under nonreducing conditions and blotted onto polyvinylidene difluoride (Millipore, Kloten, Switzerland) membranes (22). The protein bands of interest were cut out from the Immobilon filter after Ponceau S staining and processed as described previously (15). Direct *in situ* amino acid sequencing of blotted proteins was performed with an Applied Biosystems 470A gas phase sequencer/120A phenylthiohydantoin derivative analyzer system according to the manufacturer's program. An average repetitive yield of 93% of derivatized amino acids per degradation cycle was obtained from 80 pmol of protein.

**Preparation of Lipoproteins and ApoA-I.**—Lipoproteins were isolated from human plasma by standard sequential ultracentrifugation using a Beckman 50.3 Ti rotor at 4 °C and 48,000 rpm (23). LDL, HDL, and HDL subfractions were separated according to densities in KBr solutions, LDL,  $d = 1.019-1.063$ ; HDL,  $1.063-1.21$  g/ml; HDL<sub>α</sub>,  $1.063-1.125$  g/ml; HDL<sub>β</sub>,  $1.125-1.21$  g/ml. The subfractions were dialyzed against 10 mM Tris-HCl, pH 7.4, 150 mM NaCl containing 1 mM EDTA to remove the KBr and stored at 4 °C. Lipoproteins were dialyzed against 10 mM Tris-HCl, pH 7.4, 150 mM NaCl containing 1 mM EDTA to remove the KBr and stored at 4 °C. Lipoproteins were dialyzed against 10 mM Tris-HCl, pH 7.4, 150 mM NaCl containing 1 mM EDTA to remove the KBr and stored at 4 °C.

**Electron Microscopy.**—Affinity-isolated apoA-I-clusterin complexes were diluted with four volumes of 100 mM NH<sub>4</sub>OAc, 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.4, and applied to a carbon-coated grid (25). The grids were washed twice with the diluting buffer and negatively stained with uranyl formate (Eastman Kodak Co.). The micrographs were recorded at a primary magnification of 35,500 in a Philips EM 400 electron microscope operating at 100 kV.

**Lipid Analysis.**—Total cholesterol, unesterified cholesterol, triacylglycerides, and the different types of phospholipids in concentrated apoA-I-clusterin preparations were analyzed by high-performance thin-layer chromatography (HPTLC) as described previously (26, 27). 0.4 mg of apoA-I-clusterin concentrated to a volume of 50 µl was delipidated according to Bligh and Dyer (28) with chloroform/methanol (1:2, v/v), and the lipids were chromatographically separated on HPTLC silica plates as described (26, 27). Protein content of the lipoprotein preparation was determined prior to delipidation.

**Interaction of Clusterin with Lipoproteins.**—50 µl of diluted fresh EDTA-plasma was passed through a 1-ml anti-clusterin antibody column in order to immobilize apoA-I-clusterin complexes. All bind-

ing sites for clusterin were saturated as evidenced by the appearance of clusterin antigens in the breakthrough fraction. The column was washed with 10 mM phosphate, 500 mM NaCl, 10 mM EDTA, pH 7.4 (see above), until no proteins could be detected in the effluent. The column was treated with 10 mM phosphate, 150 mM NaCl, pH 7.4 (PBS), containing 0.5% Triton X-100 to remove apoA-I and lipids. Thereafter, the column was washed extensively with PBS to remove the detergent. Fresh EDTA-plasma diluted with 1 volume of PBS was applied to the column. After washing with PBS, the bound material was first eluted with PBS plus 0.5% Triton X-100 and finally with 0.2 M glycine, pH 2.5. All steps were carried out at 4 °C. Eluted proteins of the different fractions were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. Proteins were also identified by electroblotting and immunostaining with specific antibodies (see below).

**Gel Filtration Chromatography.**—70 µl of fresh fasting plasma containing 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine and 20 units/ml aprotinin (Sigma, Munich, Federal Republic of Germany) was loaded onto a Superose 6G column equilibrated in 20 mM phosphate, 0.5 mM NaCl, pH 7.4, 10 mM EDTA at 4 °C. Proteins were eluted with the same buffer at a flow rate of 0.2 ml/min at 4 °C. Fractions of 0.5 ml were collected. To detect clusterin, 100 µl aliquots were applied to nitrocellulose using a dot-blot microfiltration apparatus (Bio-Rad, Zürich, Switzerland), for apoA-I and apoB detection only 10 µl of the fractions were used. Specific antibodies to apoA and apoB (Calbiochem, Luzern, Switzerland) and ascites fluid of murine monoclonal antibodies against clusterin were diluted 1000 times in incubation buffer. Immunostaining was performed as described for dot-blot assays below.

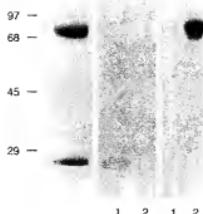
**Dot-blot Assays.**—2.5 µg of purified delipidated apoA-I, ultracentrifugation-purified HDL ( $d = 1.06-1.21$  g/ml), LDL, and bovine serum albumin were spotted onto nitrocellulose. The filter was blocked with 0.1% BSA in incubation buffer consisting of 1% gelatin, 10 mM Tris-HCl, pH 8.4, 150 mM NaCl, 5 mM EDTA. The filters were incubated with clusterin at a concentration of 2.5 µg/ml in incubation buffer for 1 h at room temperature. Binding of clusterin was detected with monoclonal antibodies II-F4 or CL1-9 and horseradish-labeled anti-mouse IgG using hydrogen peroxide and 4-chloronaphthol as the substrates.

**Analytical Isotachophoresis of Lipoproteins.**—Normal EDTA-plasma, HDL-deficient EDTA-plasma from two different Tangier patients (patients I and II in Ref. 29), and purified apoA-I-clusterin complexes were studied by analytical isotachophoresis as described (30–32). The isotachophoretic mobility of purified apoA-I-clusterin was compared with that after addition of purified apoA-I-clusterin to HDL-deficient plasma of the patients suffering from Tangier's disease (29). Absorption at 570 and 254 nm was recorded on line during separation.

**Preparative Isotachophoresis of Lipoproteins.**—Preparative separation of plasma lipoproteins was carried out in an Eiphor VAP22 (Bender & Hobein, Munich, Federal Republic of Germany) apparatus (31, 32). Fresh fasting EDTA-plasma, HDL-deficient plasma samples from the Tangier patients, and HDL samples purified by ultracentrifugation were adjusted to a final protein concentration of 1 mg/ml in terminating buffer containing spacers (32). Equal amounts of total proteins from the collected fractions were analyzed by SDS-PAGE on 12% polyacrylamide gels. After electrotransfer onto nitrocellulose membranes, clusterin was detected by immunostaining as described (1).

#### RESULTS

Affinity purification of apoA-I-clusterin complexes using the immobilized monoclonal antibody II-F4 (9) is shown in Fig. 1 (left panel). In all attempts made to remove unspecifically bound proteins with detergent-free washing buffers, a second bound protein of 28 kDa was recovered from the affinity column at pH 2.5. Therefore, a second monoclonal antibody prepared against purified clusterin (CL1-9) was used for the affinity purification. This antibody has a different binding specificity, since it binds to both nonreduced and reduced clusterin in immunoblots, in contrast to II-F4 which only recognizes nonreduced clusterin (1). However, co-purification of clusterin and the 28-kDa protein was still observed suggesting a specific association between clusterin and the 28-kDa protein. Densitometric scanning of Coomassie Blue-



**FIG. 1.** Isolation of apoA-I-clusterin complexes from human plasma. *Left panel*, co-purification of a 28-kDa plasma component with human clusterin. Approximately 10  $\mu$ g of purified proteins isolated from plasma by immunoaffinity chromatography on anti-clusterin-Sepharose was analyzed by SDS-PAGE under nonreducing conditions and the gel bands were visualized by staining with Coomassie Blue. Molecular weight markers are shown on the left. *Middle and right panel*, dissociation and elution of apoA-I from clusterin bound to anti-clusterin Sepharose with 0.5% Triton X-100 in PBS (*lane 1*) followed by elution of clusterin from the monoclonal antibody Sepharose with 0.2 M glycine, pH 2.5 (*lane 2*). Differentially eluted proteins were analyzed by SDS-PAGE and immunoblotting using a monospecific rabbit antibody against human apoA-I and apoA-II (*middle panel, lanes 1 and 2*) and a monoclonal mouse antibody against human clusterin (*right panel, lanes 1 and 2*).

stained gels showed that the amount of the isolated 28-kDa protein was about half of that for clusterin on a weight to weight basis.

To exclude that the 28-kDa protein is a specific fragment of clusterin and to determine its identity, the 28-kDa protein was separated from clusterin by SDS-PAGE under nonreducing conditions and blotted onto Immobilon for direct amino acid sequence analysis. The ten amino-terminal residues obtained are Asp-Glu-Pro-Pro-Gln-Ser-Pro-Trp-Asp-Arg and are identical to that reported for mature plasma apoA-I (33). Immunoblotting of apoA-I-clusterin complexes and HDL preparations separated by SDS-PAGE showed that the 28-kDa component indeed co-migrated with apoA-I from HDL and was also detected by antibodies to apoA-I at the same intensity (data not shown). These findings clearly indicate that the 28-kDa component represents intact apoA-I, which is a characteristic component of HDL.

We studied the nature of interaction between apoA-I and clusterin. By adding 0.5% Triton X-100 to the PBS washing buffer, we were able to completely dissociate the 28-kDa protein from clusterin. ApoA-I was quantitatively eluted in the detergent buffer (*Fig. 1, middle panel, lane 1*), whereas clusterin remained bound to the affinity column (*Fig. 1, right panel, lane 1*). The column-bound clusterin was then washed with PBS buffer and eluted with 0.2 M glycine, pH 2.5. *Lanes 2* in *Fig. 1* show that clusterin was not lost from the column during the washing steps and that it did not contain any apoA-I.

We therefore presumed that binding between apoA-I and clusterin was mediated by hydrophobic forces and that plasma lipids may be involved in the formation of HDL-like apoA-I-clusterin complexes. Electron micrographs of negatively stained apoA-I-clusterin preparations were consistent with this notion. The ultrastructural appearance of apoA-I-clusterin complexes are spherical particles the majority of which has considerable size uniformity. A small fraction of particles appears to represent dimers (e.g. see upper right corner of *Fig. 2*), multimers and aggregates and have a proportionally larger

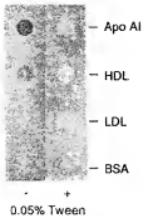


**FIG. 2.** Transmission electron microscopy of negatively stained apoA-I-clusterin complexes. ApoA-I-clusterin was isolated from fasting plasma by immunoaffinity chromatography using 0.2 M glycine, pH 2.8, as the eluent. The bar represents 50 nm.

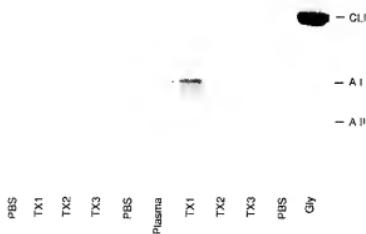
size. In addition, a fraction of smaller particles whose negative staining is weaker and sometimes irregular, are discernible on the micrograph of *Fig. 2*. The average diameter of typical well contrasted particles was measured as the distance between the negatively stained edges of individual particles. In *Fig. 2* the mean values for individually dispersed, typical apoA-I-clusterin particles was  $9.0 \pm 1.0$  nm. These values fall into the smallest size range of HDL particles, range III, 8.5–9.6 nm, as defined previously (34).

Since ultrastructural shape and size of purified apoA-I-clusterin complexes resembles that of HDL particles, we analyzed the amount and spectrum of lipids present in the apoA-I-clusterin preparations. The apoA-I-clusterin lipoprotein particle consists of 78% (w/w) protein and 22% (w/w) lipids. Cholestry ester, free cholesterol, triglycerides, and phospholipids in concentrated preparations were determined by quantitative TLC on a molar basis. The lipids were composed of 54% (mole/mol) total cholesterol, 42% phospholipids (phosphatidylcholine 85%, sphingomyelin 9%, other phospholipids 6%), and 4% triglycerides. The molar ratio between unesterified cholesterol and esterified cholesterol amounted to 0.58. Thus, the apoA-I-clusterin complex is a lipoprotein particle of unusual composition in that it has a very high protein content and is rich in free cholesterol.

The specific interaction between apoA-I free clusterin and apoA-I was tested by dot-blot assays with purified filter-bound proteins and lipoproteins in the presence and absence of detergents. 2.5 micrograms of purified apoA-I, HDL, LDL, or BSA was spotted onto nitrocellulose filter membranes. Remaining sites of the filter membrane were blocked by gelatin-containing TBS. Clusterin binding was visualized by immunostaining using monoclonal antibodies to human clusterin. We found that apoA-I free clusterin at a concentration of 2.5  $\mu$ g/ml bound to purified apoA-I and to a lesser extent also to ultracentrifugation purified HDL, but not to LDL or BSA (*Fig. 3*). This interaction was completely abolished when 0.04% Tween 20 was present in the incubation buffer. The results are consistent with the above observation that nonionic detergents dissociate clusterin from apoA-I-lipid complexes. Furthermore, the experiments suggest that clusterin specifically associates with apoA-I and apoA-I-containing



**FIG. 3.** Binding of clusterin to filter-immobilized apoA-I and HDL. 2.5  $\mu$ g of lipid-free apoA-I, lipid-free BSA, HDL, and LDL was adsorbed to nitrocellulose using a dot-blot microfiltration apparatus. After blocking with 3% albumin in TBS, filters were incubated with 10  $\mu$ g/ml clusterin from which apoA-I had been dissociated, in the absence (-) or presence (+) of 0.05% Tween 20. Bound clusterin was visualized by immunostaining using monoclonal antibodies against clusterin in combination with peroxidase-coupled rabbit anti-mouse IgG antibodies.



**FIG. 4.** Interaction of apoA-I in total plasma with Sepharose-bound clusterin. ApoA-I-clusterin lipoproteins from 50 ml of fresh fasting plasma diluted 1:1 in PBS plus 10 mM EDTA, 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine were chromatographed on a 1-ml anti-clusterin-Sepharose column. After washes with PBS (lane PBS), apoA-I (A I) and lipids were eluted with 0.5% Triton X-100 in PBS (lanes TX1-TX3). The antibody-bound clusterin was then washed with 20 volumes of PBS to remove any residual detergent. Fresh plasma diluted in PBS was then passed through the column and subsequently washed extensively with PBS. Bound proteins were eluted with detergent-containing PBS in the same way as before (lanes 7-9 from the left). After washing with PBS, clusterin (Clu) was desorbed from the column with 0.2 M glycine, pH 2.5 (lane Gly). Equal volumes of the eluted fractions were analyzed by SDS-PAGE under nonreducing conditions. Proteins on the 12% acrylamide gel were stained with Coomassie Blue.

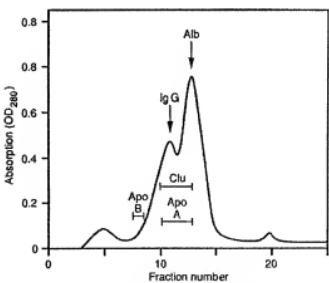
HDL particles and that hydrophobic interactions are involved in the formation of apoA-I-clusterin complexes.

To obtain further evidence for a biologically important, specific interaction between clusterin and apoA-I-lipid complexes, total plasma was passed over an anti-clusterin antibody-Sepharose column. All binding sites for clusterin were saturated by using an excess of plasma. Clusterin alone was specifically immobilized onto the affinity column by washing the affinity matrix with 0.5% Triton X-100 in PBS and then with an excess of PBS (Fig. 4, lanes 1-5 from the left). Fresh EDTA-plasma was chromatographed through the column a second time. The bound plasma proteins were eluted with 0.5% Triton X-100 in PBS (Fig. 4, lanes TX1-TX3) and then with 0.2 M glycine, pH 2.5 (Fig. 4, lane Gly). Fig. 4 shows that

apoA-I and traces of apoA-II were taken up by the immobilized clusterin from total human plasma specifically. Only small amounts of a few additional unidentified plasma proteins were detected. ApoA-I was completely removed from clusterin in nonionic detergent buffers, whereas clusterin was retained on the column. These results were verified by electroimmunoblot analysis of the different chromatographic fractions using monospecific rabbit sera to apoA (apoA-I and apoA-II) and monoclonal antibodies to clusterin (data not shown).

The native molecular mass of apoA-I-clusterin complexes in total fasting plasma was determined by gel filtration chromatography on a Superose 6B column. Previously it was reported that serum clusterin eluted from a gel filtration column in at least three different peaks, corresponding to the void volume, to the molecular mass of a monomer (68 kDa) and to the molecular mass of a dimer (8). Similar data were found for purified Sertoli cell-derived rat clusterin (SGP-2), which showed a high tendency to form dimers and large molecular weight aggregates in particular after freezing and thawing (35). Our gel filtrations experiments with fresh human EDTA-plasma yielded only one peak with clusterin immunoreactivity which eluted before albumin and slightly after immunoglobulin. High molecular weight forms of clusterin were not detected. Clusterin immunoreactivity was restricted to fractions that also contained apoA-I antigens (Fig. 5).

To exclude the possibility that the clusterin-apoA-I complex isolated by affinity chromatography is an artificial product generated by the interaction of immobilized clusterin with plasma HDL particles, we have determined the electrophoretic mobility of affinity-purified apoA-I-clusterin and of native plasma clusterin in isoelectric focusing (IEF). This technique permits one to fractionate total lipoproteins and HDL into a number of subpopulations according to the mobility of the particles in a very short period of time (10 min). Lipoproteins are prestained with the nonpolar dye Sudanblack B and are monitored at 580 nm after migration. The individual peaks



**FIG. 5.** Native molecular mass of clusterin by gel filtration of total plasma. 50  $\mu$ l of fresh fasting plasma was diluted 2-fold in PBS, 10 mM EDTA, and passed through a Superose 6B gel filtration column. The flow rate of the buffer was adjusted to 0.1 ml/min, and 500- $\mu$ l fractions were collected. Proteins in the different fractions were analyzed for apoA, apoB, and clusterin antigens by dot blotting on nitrocellulose using monospecific polyclonal anti-apoA, polyclonal anti-apoB, and monoclonal anti-clusterin antibodies. ApoB, apoA, and clusterin (Clu) eluted in the fractions designated by bars. The elution profile of plasma proteins was monitored by measuring the absorption at 280 nm. The position of human IgG and BSA (Alb) (arrows) served as calibration markers.

from total plasma have been correlated with the different classes of lipoproteins, HDL, LDL, VLDL, and IDL (31, 32). The 10 fractions collected from preparative free flow ITP of total plasma were analyzed by SDS-PAGE followed by immunoblotting. Clusterin immunoreactivity was detected in those preparative fractions, which correspond to peaks 4–6 in analytical ITP (Fig. 6, panel A) (32). In analytical runs of apoA-I-clusterin preparations most of the purified apoA-I-clusterin complexes were detected in fractions 4–6 by measuring the absorption at 254 nm as well as by detecting Sudan Black-stained lipids at 570 nm (Fig. 6, panel B, peaks b and c). A small proportion of purified apoA-I-clusterin complexes (peak a in panel B) was found in fractions 1 and 2 and showed a higher electrophoretic mobility than clusterin-HDL of human plasma. Except for this subpopulation of apoA-I-clus-

terin complexes which may form during purification or storage, purified apoA-I-clusterin had the same electrophoretic mobility as native clusterin from plasma samples indicating that the lipid-apoA-I-clusterin complex is not an artifact of affinity purification. In both samples (Fig. 6, panels A and B) clusterin co-migrated with the slow migrating subfraction III of ultracentrifugally purified HDL (peaks 4–6 in analytical ITP). Since spacing of individual lipoprotein fractions in ITP depends on the complexity of the protein sample analyzed, we also studied the isotachophoretic behaviour of clusterin-HDL in Tangier plasma samples which have a very low content of HDL. By immunoblotting ITP-fractionated plasma samples from Tangier patients, the level and isotachophoretic mobility of endogenous clusterin was found to be about the same as in normal subjects (data not shown). Since the HDL peaks 1–6 in the ITP profile of Tangier plasma lipoproteins are very low, we were able to monitor the migration of affinity-purified apoA-I-clusterin complexes (Fig. 6, peaks a–c in panel B) in analytical free flow ITP after adding them to total plasma of Tangier patients. Panel D of Fig. 6 shows that the majority of purified clusterin-HDL (panel D, peaks b and c) has the same isotachophoretic mobility as native clusterin-HDL in total human plasma (Fig. 6, panel A).

#### DISCUSSION

High density lipoproteins form a highly heterogeneous class of lipoprotein particles that interacts with a wide variety of cells and mediates various still poorly defined physiological functions. HDL levels are correlated with a low risk of premature atherosclerosis and are believed to improve the efficacy of reverse cholesterol transport. The relevant HDL subpopulations and the cellular processes underlying this anti-atherogenic protective effect of HDL have not been identified (36–38). By using anti-clusterin immunoaffinity chromatography we have isolated a distinct protein-rich lipoprotein particle which contains apoA-I, clusterin, and only 22% lipids. As the clusterin concentration in normal human plasma is about 20 times lower than that of apoA-I, apoA-I-clusterin complexes represent about 2–4% of the total apoA-I protein in plasma assuming a 1:1 or a 2:1 molar ratio. Due to its low lipid content and resulting high hydrated density relative to globular plasma proteins, the apoA-I-clusterin particle does not float with HDL or HDL-like lipoprotein species obtained by sequential density gradient ultracentrifugation. Previous reports that purified plasma clusterin exists in monomeric, dimeric, and multimeric forms (8) may be due to losses of lipids and apoA-I, partial denaturation or aggregations of apoA-I-clusterin complexes during purification. Whether uncomplexed free forms of clusterin or multimers of it are actually present in the circulation at low levels remains to be explored. The high degree of particle size homogeneity as judged by electron microscopy and gel filtration chromatography strongly supports the view that apoA-I-clusterin complexes are the predominant form of clusterin in human blood plasma. However, the molecular forms of human clusterin that are found in the brain and in the tubular lumen of the testes may be different, since these body compartments are separated from the plasma compartment by tight physiological barriers.

The biochemical properties of clusterin are typical of apolipoproteins. Lipid-free preparations of clusterin and highly concentrated preparations of apoA-I-clusterin particles (not shown) show a strong tendency to aggregate, especially after freezing (35) and are poorly water soluble. The predicted  $\alpha$ -helix content of clusterin is 41%. There are several helical segments of amphiphilic nature (residue 150–170, 215–240,

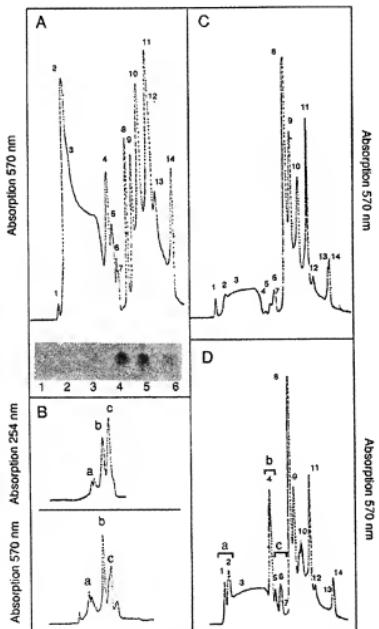


FIG. 6. Comparison of the isotachophoretic mobility of native apoA-I-clusterin complexes in fasting plasma with that of purified apoA-I-clusterin complexes. Fractions prepared by preparative free-flow ITP were analyzed by SDS-PAGE and immunoblotting (insertion of panel A) to identify those fractions containing clusterin antigens (panel A, fractions 4–6). Panel B shows the mobility by absorption measurements at 570 nm (lower curve) and at 254 nm (upper curve). Panel C shows the ITP profile of Tangier plasma lipoproteins which have low amounts of HDL in the HDL peaks 1–6. In panel D the change of the ITP lipoprotein pattern is recorded after mixing purified apoA-I-clusterin with the Tangier plasma sample analyzed in panel C. Peaks 1–6 represent HDL; peak 7, chylomicron-derived particles; peaks 8–10, VLDL, and peaks 11–14, LDL.

300–350, and 406–420) in addition to an extensive amphiphilic  $\alpha$ -helical region at the amino terminus (residue 1–76) (1) which is conserved across the human, rat, and quail species. Similar amphiphilic structures and a high  $\alpha$ -helical content are shared by all apolipoproteins, explaining the relatively high similarity scores between apoA-I and clusterin noted previously (13). Therefore, clusterin may be regarded as a new specific apolipoprotein marker of a minor HDL subpopulation.

While this manuscript was submitted for publication, de Silva and colleagues (39, 40) reported on similar findings. These authors introduced the name apoJ for clusterin. Although most of their data is in accord with those published here, there are some differences which need further consideration.

Our data on the lipid content of apoA-I-clusterin particles (22 versus 11%) differ significantly from those reported by de Silva and co-workers (39, 40). In contrast to de Silva, we did not rely on commercially available enzymatic kits to determine individual lipid classes. In our hands these kits yielded much lower values for the lipid content than the quantitative TLC procedure developed by Schmitz and co-workers in 1984 (26). The fact that the majority of apoA-I-clusterin complexes were reported to have a density in the range between 1.18 and 1.23 g/ml (de Silva's data, using sequential density gradient ultracentrifugation) is better supported by the higher lipid content of HDL-clusterin.

Comparing the micrograph of de Silva *et al.* (39, 40) with ours, the degree of microheterogeneity is apparently larger in the preparations of de Silva than in our clusterin-apoA-I samples. The authors report a total size range of 5–16 nm, whereas we find 9 nm for the diameter of a typical well dispersed and well contrasted spherical particle. The broader size range of the particles in de Silva's report presumably results from a larger proportion of denatured or aggregated material that they subjected to alkaline elution conditions during immunoaffinity purification. In our hands, stability of HDL-clusterin particles were found to be better after eluting them at pH 2.5–2.8.

In an attempt to define the protein heterogeneity of apoA-I- and apoA-II-containing HDL particles, several minor protein moieties that were not related to apoA-I, apoA-II, apoC-I, apoC-II, and apoE have been encountered by anti-apoA-I immunoadsorbent chromatography (41). These putative apolipoproteins were called NA1 to NA6 and co-purified in small amounts with apoA-I particles. NA1 to NA6 were hardly detectable in HDL purified by ultracentrifugation. On the basis of these data alone it was not clear whether the copurifying NA1 to NA6 proteins were taken up from the plasma by immobilized HDL particles unspecifically or whether NA1 to NA6 were characteristic markers for genuine minor HDL subpopulations. The amino-terminal sequence information now available for NA1 and NA2 (42) shows that NA1 and NA2 are the two disulfide-linked subunits of clusterin. Thus, the presence of small amounts of clusterin in anti-apoA-I affinity-purified HDL particles indicates that apoA-I-clusterin particles can be obtained from plasma by anti-apoA-I affinity chromatography as well as by anti-clusterin affinity chromatography as shown in this study.

In order to circumvent the perturbing effects of ultracentrifugation on HDL and HDL subpopulations, and to demonstrate that the purified apoA-I-clusterin particles represent genuine lipoproteins of native plasma, we have fractionated total plasma and purified apoA-I-clusterin lipoproteins by free-flow isotachophoresis (31, 32). Most of the purified apoA-I-clusterin lipoproteins co-migrated predominantly with the

slowest fraction of HDL (peaks 4–6) in total human plasma (32) and had the same isotachophoretic mobility as the native apoA-I-clusterin complex in a plasma sample. This finding provides further evidence that the affinity-isolated apoA-I-clusterin particle truly represents a natural lipoprotein entity in whole human plasma.

The biological properties of the different ITP fractions containing HDL have been studied recently (32). The fast and slow migrating fractions of HDL are good promoters of the cholesterol efflux from cultured cells and exhibit a high nonspecific interaction with cell surfaces, whereas the intermediate fraction lacks this activity. Since HDL subfractions isolated by ultracentrifugation have been used, the functional role of apoA-I-clusterin particles in the interaction of HDL with macrophages was disregarded. By contrast, the slow migrating HDL fraction of total plasma lipoproteins encompasses apoA-I-clusterin particles (Fig. 6, panel A). The latter may represent a biologically relevant proportion of HDL subspecies which could be involved in HDL metabolism and lipid exchange between cells and lipoproteins of the plasma and interstitial fluid (37, 38).

The capability of purified clusterin and apoA-I-clusterin lipoproteins to interact with cell surfaces is supported by the following observations. Purified clusterin of seminal plasma and apoA-I-clusterin lipoproteins induce aggregation of many different types of dispersed cells from different species *in vitro* (43). This process does not appear to depend on a specific cell surface receptor.<sup>2</sup> *In vivo*, clusterin also shows an affinity for cell membranes especially of those cells, which are damaged, abnormal, or dying (2, 44). For example, clusterin was detected on the cell surface of spermatozoa which were predominantly morphologically abnormal (2). In necrotic or involuting tissues which express very high levels of clusterin, the protein is found in association with dying cells (19, 44, 45). Clusterin and apoA-I-clusterin complexes could thus assist in the mobilization, uptake, and redistribution of lipids from damaged or lipid loaded cells.

A role for clusterin in lipid transport and local lipid redistribution among cells in peripheral tissues is further consistent with the wide distribution of clusterin and with the strong inducibility of its expression in response to mechanical, chemical, or metabolic cell damage (19). The clusterin (SGP-2) message is most abundant in testis, epididymis, liver, and brain,<sup>3</sup> low levels are detected in kidney, spleen, and the mammary gland (4, 12). Thus the tissue distribution of clusterin resembles that of apoA-I and apoE (46). In addition to this analogy, the apoA-I and apoE genes share the feature of inducibility with clusterin (16, 17, 19). ApoA-I and apoE are induced in nonneuronal bystander cells after a derenervating crush injury of peripheral nerves distal to the lesion (47, 48). Increased levels of apoE and apoA-I lipoproteins were measured in the local environment of the regenerating nerve (48). A comparable upregulation of clusterin in the nervous system has recently been observed in scrapie-infected hamster brains and in the hippocampus of Alzheimer patients (14). Increased clusterin expression may indicate ongoing repair and remodeling processes of the diseased brain tissue and could be part of a lipid redistribution process similar to that described during nerve repair in the periphery.

In view of our results, the role of clusterin in the process of programmed cell death becomes much clearer. In contrast to the hypotheses proposed previously that clusterin may directly activate an apoptotic suicide mechanism, enhance the opsonization of cell debris, or function as a chemoattractant

<sup>2</sup> B. Lowin, D. E. Jenne, and J. Tschopp, manuscript in preparation.  
<sup>3</sup> D. E. Jenne and J. Tschopp, unpublished data.

(19), the proposed role for clusterin in lipid scavenging, lipid transport, and lipid recycling is in harmony with its sequence features, with its expression in normal tissues like Sertoli cells, ependymal epithelial cells, and hepatocytes and with its unique capacity to bind plasma membrane lipids in conjunction with apoA-I. The capability of clusterin to neutralize the cytolytic potential of nascent terminal complement complexes appears to represent a second biologically important function (1, 49, 50) which may serve to minimize tissue damage in complement-mediated cytotoxic defense mechanisms.

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